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ScienceDirect
Clinical Biochemistry 43 (2010) 178–185
CLINICAL BIOCHEMISTRY

Mutation scanning of exon 20 of the *BRCA1* gene by high-resolution melting curve analysis

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Received 28 March 2009; received in revised form 25 August 2009; accepted 26 August 2009
Available online 9 September 2009

Abstract

Objectives: 5382insC frameshift mutation along with 5331G>A (G1738R) missense mutation, both found in exon 20 of the *BRCA1* gene, are relatively frequent among the Greek breast and ovarian cancer population (46%). Our goal was to develop a novel, reliable and rapid genotyping/scanning method for mutation detection of the exon 20 of the *BRCA1* gene, using high-resolution melting curve analysis.

Design and methods: The developed methodology was based on real-time PCR and high-resolution melting curve analysis in the presence of LCGreen I dye. Two amplicons on the exon 20 of *BRCA1* gene were designed (157 bp and 100 bp), one flanking the exon's boundaries, and one embracing the 5382insC mutation. Our methodology was first optimized and validated by using genomic DNA samples with the 5382insC and 5331G>A (G1738R) mutations and wild-type. In total, the developed methodology was applied on 90 peripheral blood and 127 formalin-fixed paraffin-embedded breast tissue samples.

Results: Sensitivity studies with gDNA isolated from peripheral blood showed that mutated DNA could be reliably detected in the presence of wild-type DNA at 5% and 0.5% ratio with the larger and the smaller amplicon, respectively. By using the developed methodology we successfully identified 5382insC, 5331G>A and 5370C>T (R1751X) mutations, in genomic DNA isolated from peripheral blood samples and 5382insC mutation in two breast tumors, as verified by DNA sequencing.

Conclusions: The combination of real-time PCR and high-resolution melting curve analysis provides a cost-efficient, simple and rapid approach to successfully scan exon 20 of *BRCA1* gene for these clinically important and frequent mutations.

Keywords: BRCA1; Mutations; 5382insC; Somatic mutations; Formalin-fixed paraffin-embedded tissues; High-resolution melting analysis; Real-time PCR; Breast cancer

Introduction

Germ-line mutations in the *BRCA1* and *BRCA2* genes have been associated with high-risk for the development of breast and ovarian cancer [1,2]. Several mutations in these genes have been identified as frequently occurring and some have been identified as founder mutations among different populations groups, in respect to their origin/race. On the contrary, somatic mutations are extremely rare amongst breast and ovarian cancer patients [3–8].

5382insC, found in exon 20 of the *BRCA1* gene, is the second most frequently occurring mutation [9] and we have previously identified that it accounts for 45% of the mutations found in families of Greek origin, bearing mutations in the *BRCA1* and *BRCA2* genes [10]. Another recent study suggests that mutations in exon 20 account for 56% of all mutations found in the Greek population, with 5382insC and 5331G>A (G1738R), also in the exon 20 region, being the two most frequently occurring mutations that account up to 46% amongst Greek *BRCA1/2* mutation carrier families [11]. The third most frequently reported mutation in exon 20, in this study, is 5370C>T (R1751X). Furthermore, the 5331G>A mutation has been identified as a founder mutation in Greek population along with evidence supporting pathogenicity of this mutation [12].

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doi:10.1016/j.clinbiochem.2009.08.024

Our group is involved in population screening of *BRCA* genes in Greece [10,13]. We are interested in the development of rapid and cost-efficient methods for genotyping and mutation scanning of these genes. We have recently reported a robust high-throughput method, to genotype the *BRCA1* 5382insC mutation, based on asymmetric PCR amplification and labeled specific probes, followed by melting analysis in the LightCycler [14].

However, recent findings of the mutation spectra of Greek population, along with novel technological advances, impelled us to seek out the capabilities of recently established more simple, cost-efficient, and powerful mutation analysis techniques.

High-resolution melting analysis (HRMA) is a well-established technique, which has demonstrated several advantages and capabilities for genotyping and/or mutation scanning. HRMA was initially introduced in 2003 by demonstrating the potential of amplicon melting analysis, using labeled primers, for genotyping and mutation scanning in three different genes [15]. However, the breakthrough for high-resolution (amplicon) melting analysis, after the introduction of specific instrumentation with high-resolution melting capabilities, was the utilization of intercalating saturating dyes [16]. Since then, analogous methods have been published for several genes. The first to report the use of amplicon melting analysis to genotype within the *BRCA1/2* regions were Dufresne SD et al, in 2006 [17]. Although, in that report, neither a high-resolution melting instrument, nor a saturating dye was used, the advantages and potentials of using amplicon melting analysis were well demonstrated. During the last year and with the introduction of high-resolution melting instruments widely into laboratories, four reports have been published which use HRMA for genotyping and mutation scanning of *BRCA1* and *BRCA2* genes germ-line mutations and polymorphisms [18–21].

In this study, led by the numerous advantages that the use of HRMA has to offer, we are assessing the potential of HRMA technique for genotyping two frequently occurring mutations in *BRCA1*, 5382insC and 5331G>A. In addition, we present results suggesting mutation scanning capabilities. Due to the high frequency of the 5382insC mutation among other population groups [9], a sensitive, simple and rapid HRMA method for uncontested genotyping of the 5382insC mutation was developed, amplifying a smaller region embracing this mutation. This method has many advantages over our previously reported methodology developed in the classic glass capillary LightCycler [14], which is not considered a HRMA instrument since data acquisition and thermal control is low [22]. We also describe for the first time the prospective use of HRMA for the scanning of somatic mutations of this gene in formalin-fixed paraffin-embedded (FFPE) tissues, along with sensitivity assays.

Materials and methods

Clinical samples

Genomic DNA (gDNA) was isolated from a total of 90 peripheral blood (PB) samples: 34 healthy volunteers, 44 breast cancer patients with unknown family history, and 12 samples that were previously analyzed by DNA sequencing - 6 of these samples were known to bear mutations in exon 20 (2 for 5382insC, 3 for 5331G>A and 1 for 5370C>T) and 6 were known to be wild-type in the exon 20 region - as previously reported [10,14]. Three of the wild-type (WT) and two of heterozygous (HET) gDNA samples (one for the 5382insC and one for the 5331G>A mutation), were ran as negative and positive controls respectively, in every batch. The remaining gDNA samples that were previously analyzed by DNA sequencing (mutated and WT) were added to sample batches in a random and blinded fashion.

Genomic DNA was also isolated from 127 formalin-fixed paraffin-embedded (FFPE) breast tissue samples: 91 breast carcinomas, 10 breast tumors with paired adjacent normal tissues, 10 breast fibroadenomas, and 5 histologically cancer-free specimens obtained from healthy women that underwent reduction mammoplasty. One FFPE breast carcinoma sample bearing the 5382insC mutation as verified by sequencing was included as positive control. Additional information on these patients has been previously described [23].

Genomic DNA was extracted from peripheral blood either by using the QIAamp Mini Kit (Qiagen, Germany) (24 samples) according to manufacturer's instructions or by a salt-out DNA extraction protocol (66 samples) [24]. Genomic DNA from FFPE tissue samples was extracted by using the High Pure PCR Template Preparation kit (Roche, Germany). DNA purity and quality was determined with the Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, USA).

Real-time PCR conditions

Real-time PCR was performed in the LightCycler instrument (Roche Applied Science, Germany) [22]. We have designed two sets of primers for this assay and primer sequences are demonstrated in Table 1. The first set is amplifying a 157 bp fragment with the primers flanking the exon region of exon 20 of the *BRCA1* gene, while the second set is amplifying a 100 bp fragment that surrounds the 5382insC mutation. Primers were designed using the Primer Premier 5 software (Version 5.00, Premier Biosoft International, USA), with an effort to restrain the examined mutations in each case in the centre of the PCR product (Table 1). Primers were synthesized by FORTH (Heraklion, Greece). The PCR amplicons were tested *in silico* with the POLAND program [25], and indicated a single melting transition. The WinMelt software (Version 2.0., BioRad, USA) was also employed to examine the melting profiles of mutated regions.

Real-time PCR was performed in glass capillary tubes (Roche Applied Science, Germany). The PCR reaction mix, for both sets of primers, consisted of 1X Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.05U/μL Platinum® *Taq* DNA Polymerase (Invitrogen, USA), 0.15 μg/μL BSA (Sigma, Germany), 1X LCGreen I saturating Dye (Idaho Technology, USA), 10–100 ng DNA and 0.2 μM of primers. Sterile water was used to supplement up to 10 μL.

The real-time PCR protocol for the 157 bp amplicon consisted of: one cycle at 94° for 2 min; 55 cycles of 94 °C

Table 1
Oligonucleotide sequences and properties, used in this study.

Primers	Amplicon size	Amplicon position *	Mutation position within the amplicon		
			5331G>A	5370 C>T	5382insC
HR1 F: 5′-TCC TGA TGG GTT GTG TTT GG-3′ HR1 R: 5′-TGG TGG GGT GAG ATT TTT GTC-3′	157 bp	nt 71568–71724	49	88	101
HR2 F: 5′-GAT GTG GTC AAT GGA AGA AAC CAC CA-3′ HR2 R: 5′-GGT GAG ATT TTT GTC AAC TTG AGG GA-3′	100 bp	nt 71619–71718	–	37	50

* Positions are given according to *BRCA1* genomic sequence (accession number L78833 in GenBank).

for 15 s, 65 °C for 20 s and 72 °C for 20 s; and followed by one cycle of 95 °C for 2 min with a rapid cooling to 40 °C for 2 min. The real-time PCR protocol for the 100 bp amplicon consisted of: one cycle at 94 °C for 2 min; 50 cycles of 94 °C for 15 s, 58 °C for 15 s and 72 °C for 15 s; and followed by one cycle of 95 °C for 2 min and a rapid cooling to 40 °C for 2 min. Real-time fluorescence acquisition was set at 72 °C. PCR reactions were performed in duplicate for every sample. All samples presenting mutant melting transitions were amplified for a third time all together in a separate batch.

High-resolution melting analysis

Melting was run in the HR-1 High Resolution Melter (Idaho Technology, USA) according to the following conditions; melting with data acquisition from 75 °C to 90 °C, rising with a rate of 0.3 °C/s followed by a cooling to 50 °C. By using the positive and negative control samples an optimization of the heating rate was performed, varying the ramp rate in the range of 0.02 °C/s–1.0 °C/s.

Sensitivity assays were performed by mixing gDNA samples that were heterozygous for the mutations with wild-type control gDNA samples corresponding to ratios of 0.05%, 0.5%, 5%, 10% and 25% of mutant to total dsDNA. These gDNA samples were chosen to match quantity, quality and cycle of quantification (Ct), in order to minimize PCR bias. Processed results from melting curve transitions were assessed from several individuals in a blinded fashion, marking their ability to differentiate the mutant containing DNA dilutions from the normal DNA samples.

Data processing was performed by using the software supplied with the HR-1 instrument. Data in each case were normalized to contain the melting transition domain, followed by superimposing of the melting transitions at low fluorescence percentage (2-5% of Normalized Fluorescence). First derivative and fluorescence difference plots were employed to distinguish different genotypes.

DNA sequencing

After HRMA, 7.5 μL of the PCR products (157 bp amplicon) were treated with 3 μL of ExoSAP-IT (USB, USA) according to the manufacturer's instructions. 5 μL of the treated PCR products were used as a template for cycle sequencing, using the Big Dye Terminator v1.1 kit (Applied Biosystems, USA) and the first set of primers used for amplifying the 157 bp amplicon in the PCR reaction. The reaction mix consisted of 1 μL ready reaction terminator premix, 7 μL sequencing buffer, 1 μL of primer (0.5 μM) and 6 μL of sterile water. The reactions were run on an Eppendorf Mastercycler EPGradient thermocycler (Eppendorf, Germany) according to manufacturer's instructions. The sequencing reactions were purified with NucleoSeq

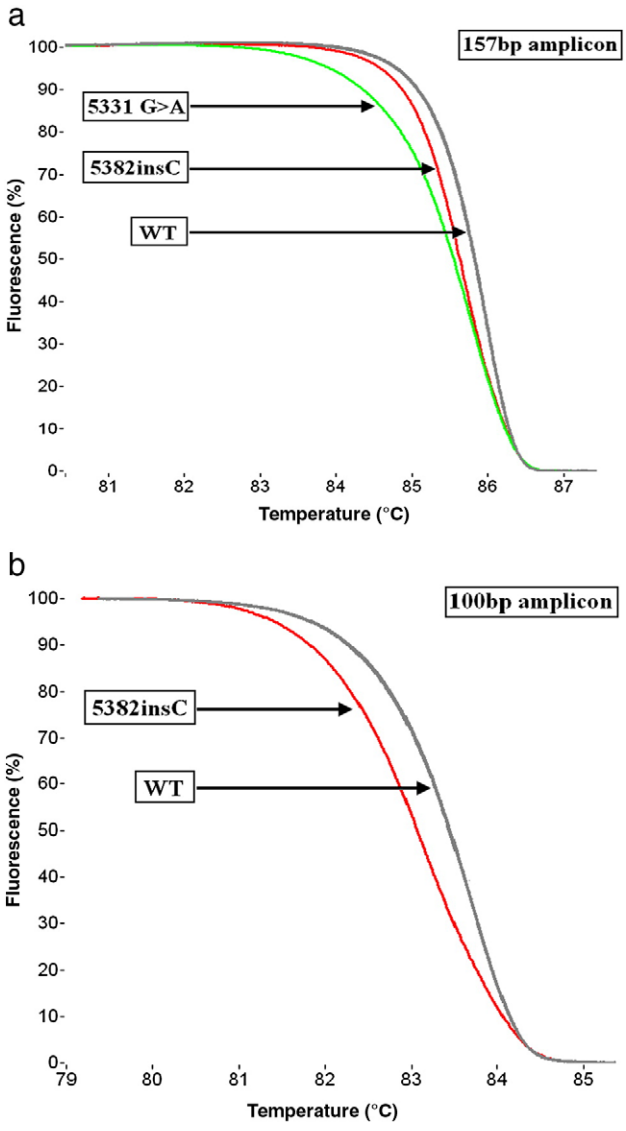

Fig. 1. Normalized melting curves of the control samples (wild-type, 5382insC and 5331G>A), of: (a) the 157 bp amplicon, and (b) 100 bp amplicon, of *BRCA1* gene exon 20.

Columns (Macherey-Nagel, Germany) and ran on an ABI 310 Genetic Analyser (Applied Biosystems, USA).

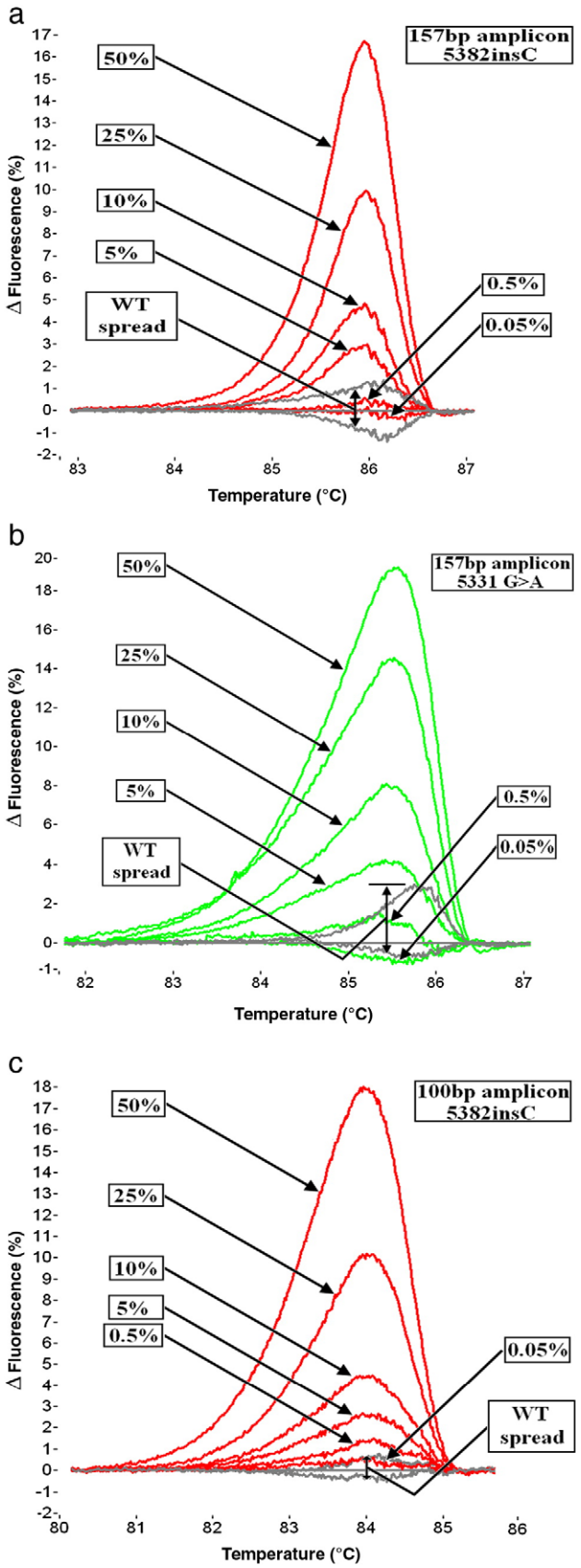

Fig. 2. Sensitivity study of HRMA for detecting *BRCA1* exon 20 mutations by Fluorescence Difference plots: (a) the 5382insC, (b) the 5331G>A mutations, using the 157 bp amplicon, and (c) the 5382insC mutation, using the 100 bp amplicon.

Results

Assay validation

To validate HRM methodology, for the 157 bp amplicon three negative (WT) and one positive heterozygous gDNA sample for each of the 5382insC and 5331G>A mutations, as previously verified by DNA sequencing, were used as negative and positive controls. As can be seen in Fig. 1a, mutant heterozygote samples were readily discriminated from WT samples using melting curves, as well as using fluorescence difference and first derivative plots (data not shown). No shoulder formation was present at first derivative plots of the 157 bp amplicons, as expected for heterozygous samples. However, mutant derivative peaks were broader and shifted to the left (after superimposing). Furthermore, differentiation between the two mutations included in this fragment was achieved, as expected by the *in silico* study results, suggesting two different regions of thermal stability (Supplementary Data, Fig. S1). Variants in domains with lower melting profile result in a melting transition, for heterozygotes, apparent in low temperature transitions. On the contrary, high temperature melting domains, exhibit a difference in melting transition at higher temperatures [26]. Thus, the HET sample for the 5331G>A mutation, having a lower region of thermal stability, produced an earlier melting transition from WT and the HET sample for the 5382insC mutation. In addition, the heteroduplex produced by the 5382insC insertion, does not correspond to an authentic mismatch. Therefore, a transition at higher temperature was expected, in contrast to a possible authentic mismatch in the region.

For the 100 bp amplicon three negative (WT) and one positive heterozygous gDNA samples, for 5382insC mutation, were used, as negative and positive controls. As can be seen in Fig. 1b, melting curves of WT and 5382insC HET samples were readily discriminated. An apparent discrimination of WT and 5382insC HET samples was achieved by using fluorescence difference and first derivative plots (data not shown). A shoulder formation was present at the first derivative plot of 5382insC mutation.

Heating rate optimization

Differences in melting transitions in respect to different heating rates have previously been reported, where it has been shown that high heating ramp rates may favor the generation of heteroduplex peaks when first derivative plots are employed [15]. We evaluated the effect of heating rate for both amplicons. Using the derivative plots, for the 157 bp amplicon, no apparent shoulder formation was observed in any of the mutant melting transitions (data not shown). Whereas, for the 100 bp amplicon a shoulder formation was observed from heating rates of 0.3 °C/s and lower (Supplementary Data, Fig. S2). The left peak,

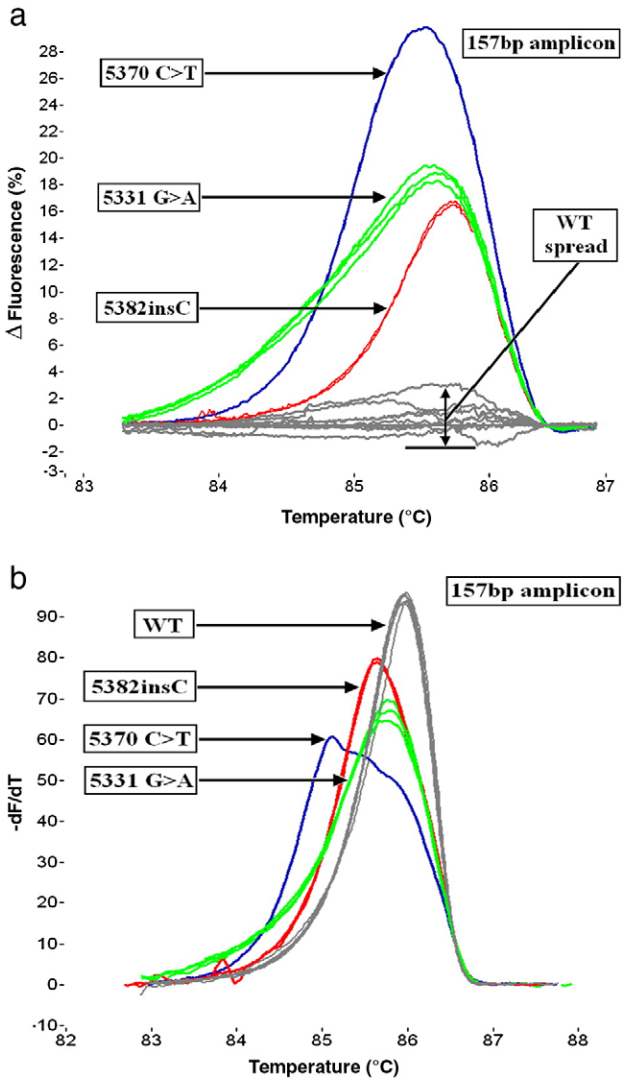

Fig. 3. HRMA detection (157 bp amplicon) of *BRCA1* exon 20 mutations in genomic DNA isolated from peripheral blood samples. (a) Fluorescence Difference plot and (b) First Derivative plot.

attributed to the heteroduplex denaturation, is emerged and increased at higher rates. In heating rates higher the 0.3 °C/s the left peak covers the right peak, which is attributed to the homodublex denaturation. In all cases, the WT amplicons presented a single melting domain, and presented sharper peaks than mutants.

Using the fluorescence difference plots, mutated samples were easily discriminated from WT samples, and from each other, in all of the heating ramp rates that were tested.

Sensitivity assay

Sensitivity is an important factor when scanning for somatic mutations in tumor tissues, since the tumor tissue could be contaminated with adjacent normal tissue. We evaluated the sensitivity of the developed methodology in order to scan for somatic mutations. Dilutions of 0.05%, 0.5%, 5%, 10% and 25% of mutated dsDNA isolated from peripheral blood, of the 5382insC HET and 5331G>A HET control samples to WT samples were applied. Fluorescence Difference plots were employed in this case, since differentiation of the various dilutions from the WT baseline was visually more apparent. As can be seen in Figs. 2a and b, by using the 157 bp amplicon, the developed method could clearly detect mutated dsDNA down to 5% for both 5382insC and 5331G>A. As can be seen in Fig. 2c, for the 100 bp amplicon, the developed method could readily detect as low as 0.5% for the 5382insC mutation.

BRCA1 exon 20 mutation detection in genomic DNA samples isolated from peripheral blood

We applied the developed HRMA methodology for both the 157 bp and 100 bp amplicons in 90 gDNA samples isolated from peripheral blood from 34 healthy volunteers, 44 breast cancer patients, and 12 gDNA samples with known sequences. These samples were extracted by using two different extraction protocols with the DNA samples ending up in different buffer

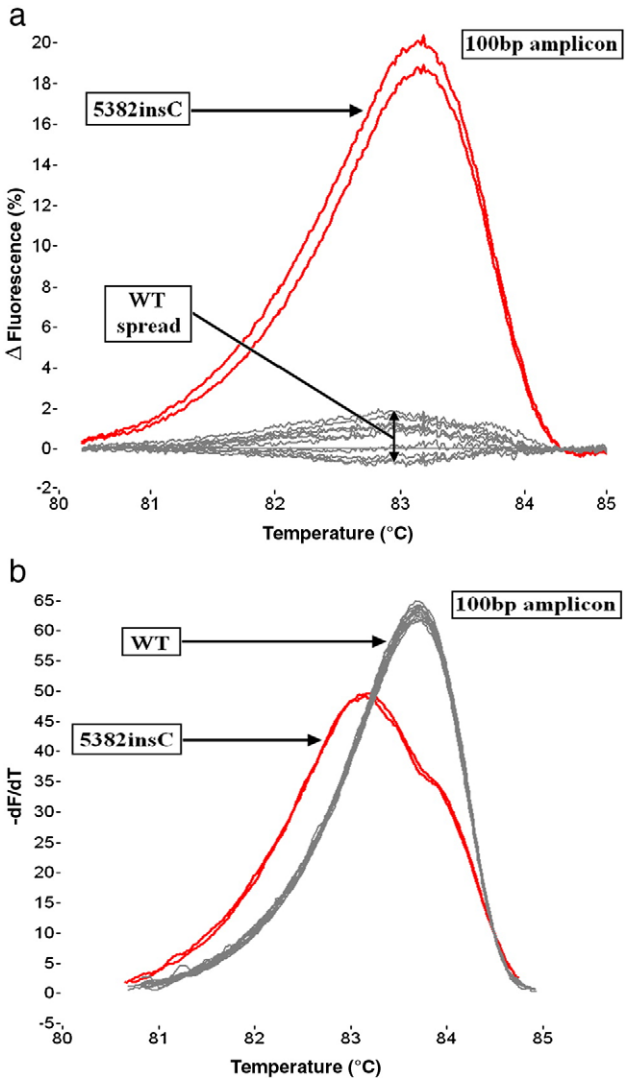

Fig. 4. HRMA detection (100 bp amplicon) of 5382insC of *BRCA1* exon 20 mutation in genomic DNA isolated from peripheral blood samples. (a) Fluorescence Difference plot and (b) First Derivative plot.

solutions. However, no significant variations were observed between the melting transitions of these two groups.

When amplifying the 157 bp amplicon, all samples with alterations verified by DNA sequencing, were successfully genotyped, using either the fluorescence difference or derivative plots, in respect to the used positive controls (5382insC and 5331G>A), (Figs. 3a and b). By using the 157 bp amplicon we were also able to detect the 5370C>T mutation, since it was easily discriminated from the WT samples as well as from the rest of the mutated samples, presenting a unique melting transition. This mutation was located at a well-centered position (position 88) of the 157 bp amplicon.

By using the 100 bp amplicon, the sample known to bear the 5382insC mutation was readily genotyped, either with fluorescence difference plots or first derivative plots (Figs. 4a and b). The larger WT spread observed for the 100 bp amplicon in Fig. 4a as compared to the WT observed in Fig. 2c is due to the fact that it is derived from a much larger number of clinical samples used in this case. Melting transitions of all samples, wild-type and mutant, produced strong clustering according to their genotype. However, by using the 100 bp amplicon we could not detect the 5370C>T mutation, present in the amplified region. In contrast to the 157 bp amplicon, where the 5370C>T mutation was well centered on the amplicon (Table 1), this mutation was located at the 37 position of the 100 bp amplicon. This might be the critical difference, for not being able to detect this mutation with the 100 bp amplicon.

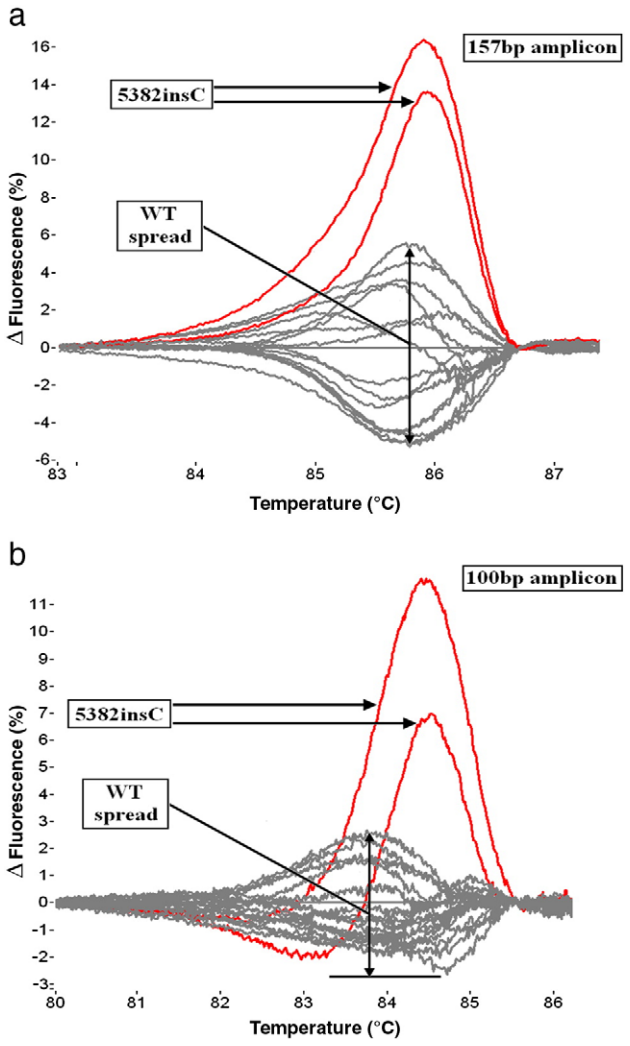

Fig. 5. HRMA detection of *BRCA1* exon 20 mutations in FFPE samples using Fluorescence Difference plots (a) 157 bp amplicon, and (b) 100 bp amplicon.

BRCA1 exon 20 mutation detection in FFPE tissue samples

We have applied the developed HRMA methodology using both the 157 bp and 100 bp amplicons to scan for *BRCA1* exon 20 somatic mutations in 127 FFPE breast tissue samples. All of the 15 normal and 10 fibroadenoma samples analyzed presented WT transitions. Out of the 102 breast tumor FFPE samples analyzed, one that was previously known to be heterozygous for 5382insC was successfully identified. In addition, one FFPE sample was found to bear a mutant transition and was further identified by DNA sequencing as a heterozygote for 5382insC. These samples were successfully identified by using both amplicons. The mutated samples could be clearly identified when using either fluorescence difference plots (Figs. 5a and b) or first derivative plots (data not shown). Two samples presenting WT melting transitions were also verified by DNA sequencing.

As can be seen in Figs. 5a and b, when analyzing FFPE samples, a significant increase in the WT baseline variation was observed, in respect to the WT baseline observed when gDNA samples isolated from peripheral blood were used (Fig. 4a). In this case, when analyzing FFPE samples for the small amplicon we estimate that the method can detect the presence of 5% mutated sequence. Since the baseline variation was more intense when using the 157 bp amplicon, we estimate that in this case the method can detect the presence of 10% mutated sequence. Mutant transitions from FFPEs were not similar with the corresponding ones taken from PB gDNA samples. However, they were easily discriminated from wild-type transitions.

Discussion

The aim of our study was to evaluate the capabilities of high-resolution melting analysis as a high-throughput method for genotyping and mutation scanning for exon 20 of the *BRCA1* gene in gDNA isolated from peripheral blood and FFPE tissues. By applying HRMA on exon 20 of the *BRCA1* gene we cover more than 50% of the *BRCA* mutated samples according to the mutation spectrum in the Greek population. We also applied the developed methodology in FFPEs, extracting some important conclusions to be considered when working with such samples.

According to our results, both fluorescence difference and derivative plots could be used for genotyping and scanning. Samples bearing mutations, for which positive controls were available and used, were genotyped successfully after comparison of their transitions. By using the 157 bp amplicon, we could also readily detect the 5370C>T (R1751X) mutation, which is also present in the Greek population [10,11]. By using the 100 bp amplicon we could more powerfully genotype the

5382insC mutation. The superiority of the smaller amplicon to detect the 5382insC mutation was more obvious when the first derivative plots were employed, due to the shoulder formation and the broadness of the first derivative curve, in contrast to the 157 bp amplicon (Figs. 3b and 4b).

It must also be noted that the PCR amplification cycles were adjusted at a relatively high number as indicated in Materials and Methods, due to the observation, during the optimization experiments, that when a mutant sample was readily amplified (favorably to reach plateau) the melting transition simulated better the transition of the control sample, with better clustering of the melting transitions. On the contrary, when the sample did not reach plateau, minor differences were observed. In the case of the 100 bp amplicon the shoulder was not apparent, in First Derivative plots, when samples were not readily amplified (Supplementary Data, Fig. S3). Similar observations in FFPE samples have been previously reported, and an increase in PCR amplification cycles up to 60 assisted in overcoming this drawback [27].

When, scanning for somatic mutations, two tumor samples bearing the 5382insC mutation were detected out of the 102 (2%) breast carcinomas analyzed. However, the melting transition from FFPEs did not accurately simulate the melting transitions of gDNA isolated from PB control samples. Such a phenomenon has previously been reported in FFPE samples when using HRMA [27], as well as, when using other techniques [14]. We also observed an increase at the baseline variation from melting curves obtained by FFPEs, in contrast to PB, which was more intense when the larger amplicon (157 bp) was used. In this case, when analyzing FFPE samples for the small amplicon the method can detect the presence of 5% mutated sequence, while for the larger amplicon the method can detect the presence of 10% mutated sequence. Increasing baseline variation has also been previously reported in FFPE samples [28]. Apart from one tumor sample which we identified as heterozygous for the 5382insC mutation, we did not detect any other mutated sample in the FFPE samples that we analyzed. However, this mutation could not be characterized as somatic, since neither normal adjacent tissue nor peripheral blood for this patient was available.

Several mutation detection techniques have been reported, with DNA sequencing considered as the reference method. However, DNA sequencing has an established sensitivity of 20% in good quality gDNA samples, and the best sensitivity for DNA sequencing reported so far for *BRAF* gene common mutation is 5% [29]. High sensitivity is an important factor when scanning for somatic mutations, since tumor samples could be contaminated with adjacent normal tissue. With the developed method, in FFPE samples 5% sensitivity was achieved when using the 100 bp amplicon for the 5382insC mutation, while a 10% sensitivity was achieved with the 157 bp amplicon for both 5382insC and 5331G>A mutations. This demonstrates an advantage for using the smaller amplicon, in respect to the capability of detecting lower proportions of mutant to wild-type dsDNA. In addition the smaller amplicon demonstrated a lower baseline variation, and increased PCR amplification efficiency.

This is the first report utilizing HRMA to scan for somatic mutations, in FFPE samples, within the *BRCA1* region, along with a sensitivity study. We are also the first to report the detection of 5370C>T mutation using HRMA. The developed HRMA methodology for *BRCA1* exon 20 mutation analysis is cost-efficient, since no specific hybridization probe for either the WT or MUT allele is needed. This is a major advantage of this approach given that there is no need for expensive and specific reagents other than an intercalating saturating dye and the corresponding set of primers. It is also rapid, 2–2.5 h per 30 samples (~1 h for the PCR reaction and ~1–1.5 h for HRMA) and very simple to perform. The developed methodology is performed in a closed-tube format, and is non-destructive. The glass capillaries are transferred from the LightCycler directly to the HR-1 HRM instrument after PCR with no further processing, since the classic glass capillary LightCycler is not considered a HRMA instrument [22]. Real-time PCR instruments are commonly found in most Molecular Diagnostics Laboratories today, while real-time PCR instruments with HRMA capabilities are commercially available.

The HR-1 instrument used in this study is a very cost-effective solution especially when the number of samples to be analyzed is relatively low. Moreover, since our optimization experiments were performed to reach plateau in PCR, a conventional PCR thermal cycler could also be used prior to HRM analysis in the HR-1 instrument. The developed methodology can also be applied without any modification in a high-throughput analysis format, by using 96-well or 384-well plate-based commercially available instrumentation, like the LightCycler 480 (Roche), and the newly released LightScanner 32 (Idaho technology).

In conclusion, the developed methodology based on the combination of real-time PCR and high-resolution melting curve analysis provides a reliable, cost-efficient, simple and rapid approach to successfully scan exon 20 of *BRCA1* gene for these clinically important and frequent mutations.

Acknowledgments

This study was funded by the Special Research Account of the University of Athens, Greece. We would like to thank Dr. N. Mavroides (Bioanalytica SA, Greece) for technical support, Dr. D. Christou (Bioanalytica SA, Greece) for financial support and Prof. V. Georgoulias (Medical School, University of Crete) for providing the clinical samples used in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2009.08.024.

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